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Molecular Cloning and Characterization of Human *FGF-20* on Chromosome 8p21.3-p22

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The fibroblast growth factors (FGFs) play important roles in morphogenesis, angiogenesis, tissue remodeling, and carcinogenesis. Human *FGF-20* has been cloned and characterized in this study. *FGF-20* encodes a 211-amino-acid polypeptide with the FGF-core domain. A strong hydrophobic region was found in the FGF-core domain of FGF-20; however, no typical N-terminal signal sequence was found in FGF-20, just as in FGF-9 and FGF-16. Total amino acid identities are as follows: FGF-20 vs FGF-9, 71.6%; FGF-20 vs FGF-16, 66.2%; FGF-9 vs FGF-16, 72.4%. Phylogenic analysis indicated that FGF-20, FGF-9, and FGF-16 constitute a subfamily among the FGF family. *FGF-20* mRNA of 2.4 kb in size was detected in colon cancer cell line SW480 by Northern blot analysis. Lower levels of *FGF-20* mRNA were detected in human fetal tissues and primary cancers by cDNA-PCR. The nucleotide sequence of *FGF-20* cDNA is split into three parts in the human genome sequence of the chromosome 8p21.3-p22 region (Accession No. AB020858). These results indicate that the *FGF-20* gene, located on human chromosome 8p21.3-p22, consists of three exons. Compared with the nucleotide sequence of *FGF-20* cDNA determined in this study, one nucleotide deletion and one nucleotide substitution in the putative coding region were identified in human genome sequence AB020858. © 2000 Academic Press

Key Words: FGF; colorectal cancer; chromosome 8p21.3-p22.

The fibroblast growth factors (FGFs) are involved in a variety of physiological and pathological processes, including morphogenesis, angiogenesis, tissue remodeling and carcinogenesis (1–3). All FGFs so far reported share a conserved "FGF core domain" of ~120 amino acids with a common tertiary structure. FGFs are exported from the expressing cells, and are trapped by heparan sulfate proteoglycans (HSPGs) on the cell surface and in the extracellular matrix (4–6). HSPGs facilitate the association between FGFs and high-affinity FGF receptors (FGFRs), which are receptor type of tyrosine kinases with extracellular immunoglobulin-like domains (7–9). FGF binding to FGFR induces dimerization and transphosphorylation of FGFRs, which leads to the activation of the small GTPase Ras and the activation of phosphoinositide 3-kinase (PI 3-kinase) (2, 10).

Among the FGF family, FGF-1/aFGF and FGF-2/bFGF were isolated as mitogens for fibroblasts from the pituitary and brain (11, 12). FGF-3/Int-2, FGF-4/HST-1, FGF-5, FGF-6/HST-2 were isolated as oncogenes (13–16). FGF-7/KGF, FGF-8/AIGF, FGF-9/GAF were isolated as mitogens for culture cells (17–19). FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, FGF-16, FGF-17, FGF-18, and FGF-19 were identified by degenerate polymerase chain reaction (PCR) (20–25). FGF-15 was identified as a downstream target of a chimeric homeodomain oncoprotein (26).

Recently, *XFGF-20* of *Xenopus laevis* was reported to be isolated depending on the homology to *XFGF-9* (27). *XFGF-20* is zygotically expressed during *Xenopus* embryogenesis, and selectively expressed in the stomach and testis of adults. In this paper, molecular cloning and characterization of human *FGF-20* will be described.

MATERIALS AND METHODS

Computer biology. By using the nucleotide sequence of *XFGF-20* (27) as bait, human genome sequence homologous to *XFGF-20* was searched for with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) as previously described (28). The candidate human genome sequence was analyzed with the GENETYX-MAC 10.1 software.

The nucleotide sequence of human *FGF-20* will appear in the DDBJ/EMBL/GenBank databases under Accession No. AB044277.

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TABLE 1
List of PCR Primers

Primer	Nucleotide sequence	Nucleotide position
PFG20-01	TGCCATTTCAGTGCAAAGTC	−26 to −7 of *FGF-20*
PFG20-02	CTTCCATAATGTCACTATCGCAC	793 to 771 of *FGF-20*
PFG20-03	AGCGACCTCAGAGGAGTAAC	1 to 20 of *FGF-20*
PFG20-04	GACCTGGCGCCATCTCTTG	675 to 657 of *FGF-20*
PFG20-05	GCAGCTCTATTGCCGCACCG	334 to 353 of *FGF-20*
PFG20-06	GTCTACTGGTAAGGACTAATC	1016 to 996 of *FGF-20*
PFG20-07	GTGCGATAGTGACATTATGGAAG	771 to 793 of *FGF-20*

cDNA-PCR. cDNA-PCR was performed as previously described (29). PCR primers corresponding to the putative exons of the human *FGF-20* gene as well as the human *β-actin* cDNA are listed in Table 1. Fifty nanograms of total RNA was used as a template of One-Step RT-PCR Kit (QIAGEN). Cycle profile of cDNA-PCR with 1.0 μM each of *FGF-20* primers was as follows: reverse transcription at 55 °C for 30 min; initial PCR activation at 95 °C for 15 min; 35 cycles of amplification, 95 °C for 0.5 min, 60 °C for 0.5 min, 72 °C for 2 min; and final extension at 72 °C for 10 min. PCR products were purified with QIAEX Kit (QIAGEN), and were ligated to pCR2.1 vector (Invitrogen). Plasmid DNAs were extracted with Plasmid Mini Kit (QIAGEN) for nucleotide sequencing analysis with ABI PRISM 310 (PE Applied Biosystems).

RNA extraction. Total RNAs were extracted from surgical specimen of primary gastric cancer with ISOGEN (Nippon Gene). Poly(A)+RNAs were extracted from gastric cancer cell lines OKAJIMA, TMK1, MKN7, MKN28, MKN45, MKN74, and KATO-III (30, 31) with Fast Track 2.0 Kit (Invitrogen).

Northern blot analyses. Two micrograms of poly(A)$^+$ RNAs extracted from indicated sources was separated by 1.0% agarose gels containing 6.6% formaldehyde in 1× Mops buffer, and were transferred onto a NitroPure nitrocellulose filter (OSMONICS), and then were fixed by baking at 80 °C for 2 h in a vacuum oven as previously described (32). Northern blot filters were hybridized with a [α-^{32}P]dCTP-labeled probe in the QuikHyb hybridization solution for 1 h (Stratagene). After washing in 2× SSC buffer containing 0.1% SDS at room temperature for 15 min twice, and in 0.1× SSC buffer containing 0.1% SDS at 60 °C for 30 min, Northern filters were exposed to IMAGING PLATE BAS-III (FUJI) for imaging analysis with STORM 820 (Molecular Dynamics).

RESULTS

Identification of the putative human FGF-20 gene. The human genome sequence homologous to *Xenopus laevis XFGF-20* was searched for with the BLAST program, and the human genome sequence of the chromosome 8p21.3-p22 region (Accession No. AB020858) was identified to be homologous to *XFGF-20*. The nucleotide position 16,020–15,930 of the human genome sequence AB020858 was homologous to the nucleotide position 698–788 of the *XFGF-20* cDNA (87% nucleotide identity), and also the nucleotide position 7496–7288 of the human genome sequence AB020858 was homologous to the nucleotide position 896–1104 of the *XFGF-20* cDNA (92% nucleotide identity).

Exon 1 of the putative human *FGF-20* gene was assumed to be successive to the nucleotide positions of 16,020–15,930 of AB020858 in the reverse direction, and the complementary nucleotide sequence of AB020858 was translated into amino acids in three frames. An amino acid sequence MAPLAEVGGFLGGLEGLGQQVGSHFLLPPAGERPPLLGERRSAAERSARGGPGAAQLAHLHGILR was found to be translatable from the region spanning the nucleotide position 16214–16020 of AB020858.1, which showed 54.9% nucleotide identity to the *XFGF-20* cDNA, and 60% amino acid identity to the XFGF-20 polypeptide.

Isolation of human FGF-20 cDNAs. Depending on the nucleotide sequence of the human chromosome 8p21.3-p22 region (Accession No. AB020858), PCR primers were synthesized. First-round PCR with primers PFG20-01 and AP1, followed second-round PCR with primers PFG20-03 and PFG20-02, amplified FG20A cDNA of 793 bp in size from Marathon-Ready cDNA of SW480 cell line (CLONTECH). cDNA-PCR with primers PFG20-05 and PFG20-04 amplified FG20B cDNA fragment of 342-bp in size from a mixture of poly(A)+RNAs extracted from gastric cancer cell lines. In addition, cDNA-PCR with primers PFG20-07 and PFG20-06 amplified FG20C cDNA fragment of 246-bp in size from poly(A)$^+$ RNAs extracted from SW480 cell line (Fig. 1A).

Overlapping *FGF-20* cDNAs spanning 1016 nucleotides consist of 5′-UTR of 133 bp in length, an open reading frame of 636 bp in length, and 3′-UTR of 247 bp in length (Fig. 1A). The initiation codon is preceded by an in-frame stop codon and the Kozak's consensus sequence for initiation of translation (Fig. 1B).

Amino acid sequence analysis of FGF-20 polypeptide. The *FGF-20* gene encodes 211-amino-acid polypeptide with the FGF-core domain (Fig. 1B). Molecular weight of FGF-20 polypeptide was calculated to be ~23 kDa. Met^1, Leu^4, Pro^{28}, Gly^{62}, Leu^{69}, Tyr^{70}, Gly^{74}, Leu^{77}, Gly^{83}, Leu^{98}, Val^{108}, Ile^{110}, Tyr^{118}, Met^{121}, Gly^{125}, Leu^{127}, Cys^{137}, Phe^{139}, Glu^{141}, Tyr^{148}, Tyr^{151}, Ser^{153}, Gly^{173}, Gly^{178}, Phe^{190}, and Pro^{192} are conserved among other members of the FGF family (Fig. 1B). No potential N-glycosylation site (Asn-X-Ser/Thr) was found in the amino-acid sequence of FGF-20. Kyte & Doolittle hydropathy analysis on FGF-20 polypeptide showed a strong hydrophobic region in the FGF-core domain.

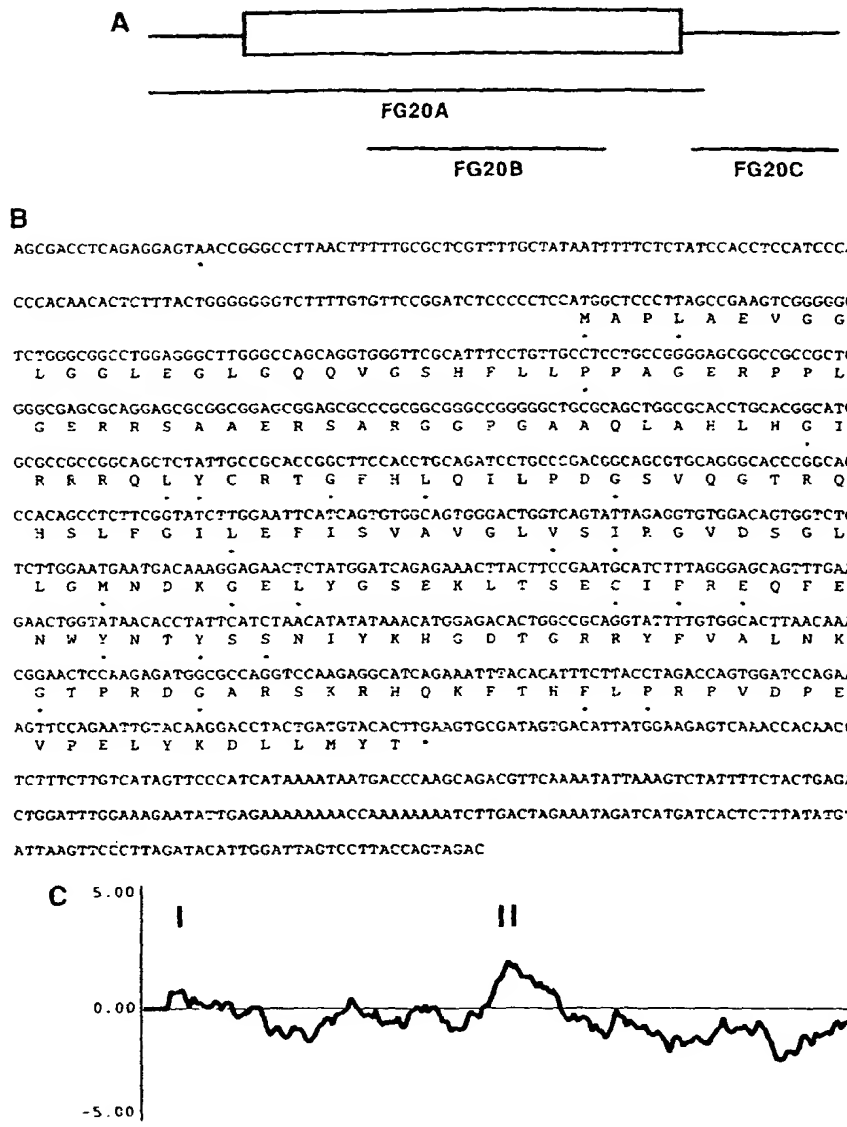

FIG. 1. Structure of *FGF-20* cDNA and FGF-20 polypeptide. (A) Schematic representation of *FGF-20* cDNAs. The coding region is depicted as an open box, the 5'-UTR and the 3'-UTR as solid bars. FG20A cDNA corresponds to the nucleotide position 1–793. FG20B cDNA corresponds to the nucleotide position 334–675. FG20C cDNA corresponds to the nucleotide position 771–1016. (B) Nucleotide sequence and deduced amino-acid sequence of FGF-20. Deduced amino-acid sequence is indicated below the nucleotide sequence. Nucleotides and amino acids are numbered on the right. The stop codon and an in-frame stop codon in the 5'-UTR are also shown by asterisks. Conserved amino acids of the FGF-family members are indicated by dots below amino-acid. (C) Kyte & Doolittle hydropathy analysis. A strong hydrophobic region in the FGF-core domain (II), and a weak hydrophobic region at the N-terminus (I) are identified in the FGF-20 polypeptide.

and a weak hydrophobic region at the N-terminus; however, no typical N-terminal signal sequence was found in FGF-20 polypeptide (Fig. 1C).

The phylogenic tree of twenty members of the mammalian FGF family indicates that FGF-20 is most homologous to FGF-9, and also that FGF-20, FGF-9 and FGF-16 constitute a subfamily among the mammalian FGF family (Fig. 2A). Total amino-acid identity between FGF-20 and FGF-9 is 71.6% (Fig. 2B), and amino-acid identity between FGF-20 and FGF-9 in the FGF core domain is 76% (Fig. 2C).

Expression of FGF-20 mRNA. The level of *FGF-20* mRNA in various human tissues and human cancer cell lines were investigated. *FGF-20* mRNA of 2.4 kb in size was detected only in colon cancer cell line SW480, but not in normal adult or fetal tissues by Northern blot analyses with the FG20C cDNA as a probe (data

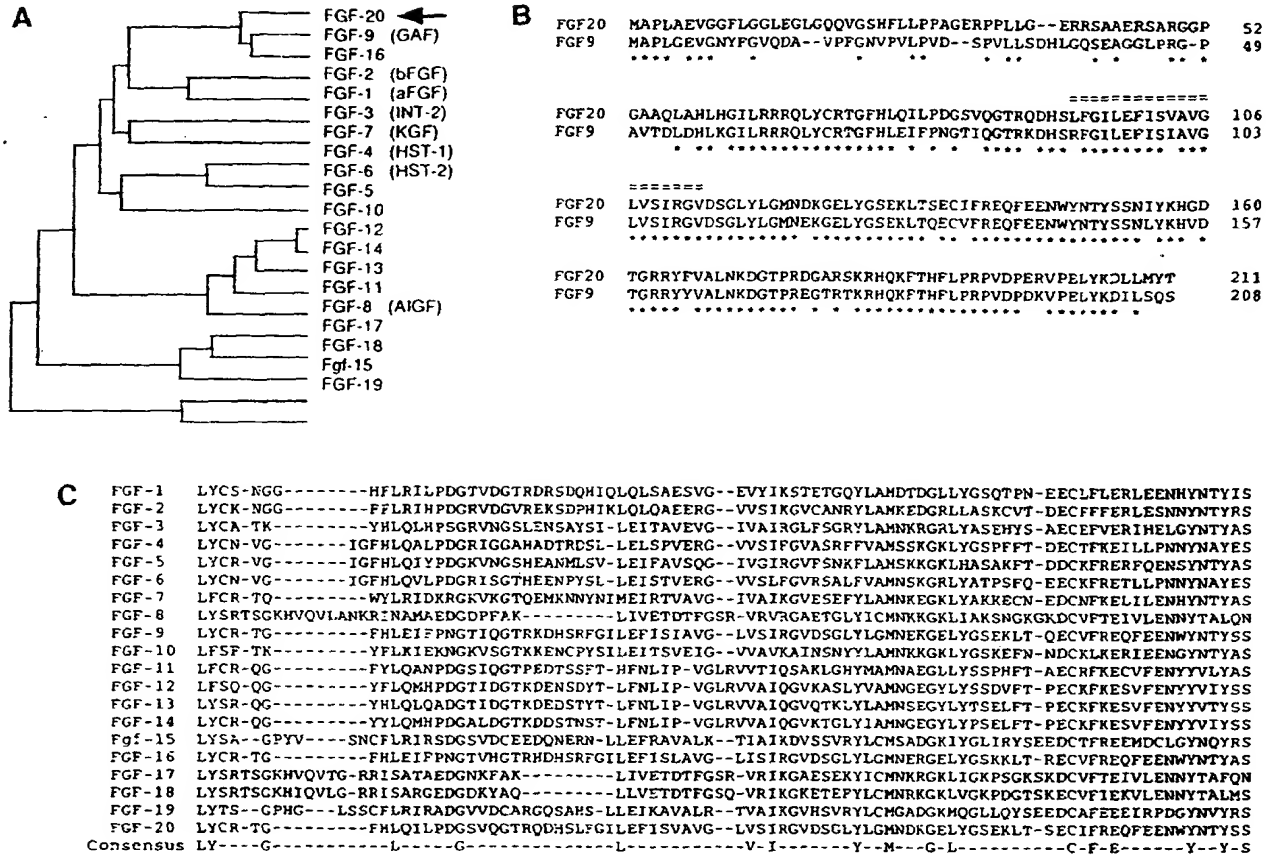

FIG. 2. (A) Phylogenic tree of mammalian FGFs. FGF-15 is derived from mouse, while other FGFs are derived from human. It is apparent that FGF-20, FGF-9, and FGF-16 constitute a subfamily. (B) Comparison between FGF-20 and FGF-9. Amino acids are numbered on the right. A strong hydrophobic region in the FGF-core domain is shown by double overline. Identical amino acid is shown by asterisk below the alignment. (C) Amino-acid alignment of FGFs in the FGF-core domain. Indicated below the alignment is the consensus amino-acid sequence in the FGF-core domain, which are defied as amino acids conserved in more than 85% of the FGF family members.

not shown), as well as by Northern blot analyses with the FG20B cDNA as a probe (Fig. 3).

Although FG20B cDNA was amplified from a mixture of poly(A)$^+$ RNAs extracted from gastric cancer cell lines, *FGF-20* mRNA was not detected in each gastric cancer cell lines by Northern blot analyses (data not shown).

By cDNA-PCR with primers PFG20-07 and PFG20-06, FG20C cDNA was predominantly amplified from poly(A)+RNAs of SW480 cells compared with those of human fetal brain, human fetal liver and human fetal kidney as well as human gastric cancer cell lines (data not shown).

Comparison between FGF-20 cDNA and genome sequence AB020858. The nucleotide position 1–419 of the *FGF-20* cDNA corresponds to the nucleotide position 16347–15930 of the human genome sequence AB020858. The nucleotide position 420–523 of the *FGF-20* cDNA corresponds to the nucleotide position 9941–9838 of the human genome sequence AB020858. The nucleotide position 524–1016 of the *FGF-20* cDNA corresponds to the nucleotide position 7499–7007 of the human genome sequence AB020858. C at the nucleotide position 277 of *FGF-20* cDNA was found to be substituted to G in the human genome sequence AB020858, and C at the nucleotide position 329 of *FGF-20* cDNA was found to be deleted in the human genome sequence AB020858.

These results indicated that the *FGF-20* gene consists of three exons (Fig. 4A). Exon 1 and exon 2 were interrupted by 5988 bp of intron 1, while exon 2 and exon 3 were interrupted by 2338 bp of intron 2. The consensus sequence of splice donor and acceptor sites (33) were found at the exon-intron boundaries of the *FGF-20* gene (Fig. 4B).

Nucleotide sequence analysis of the FGF-20 gene in human cancer. As the *FGF-20* gene is located on human chromosome 8p21.3-p22, where loss of heterozy-

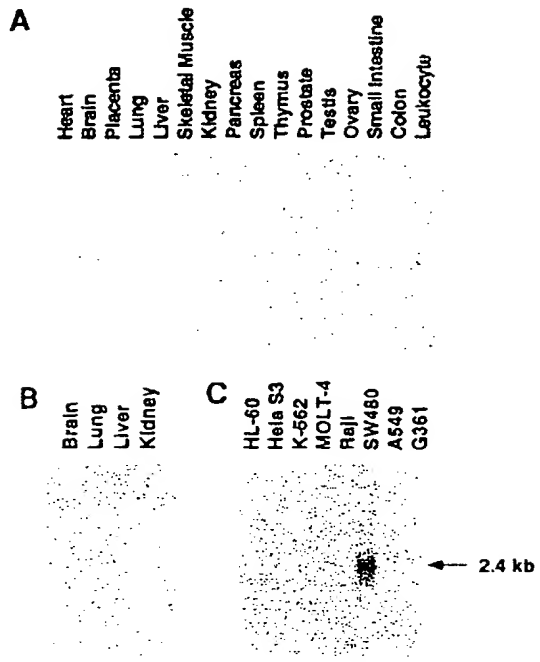

FIG. 3. Northern blot analyses on *FGF-20* mRNA expression. The level of *FGF-20* mRNA in various human tissues and human cancer cell lines were investigated by Northern blot analysis with the FG20B probe, corresponding to the nucleotide position 334–675. (A) Adult human tissues. (B) Fetal human tissue. (C) Human cancer cell lines. *FGF-20* mRNA of 2.4 kb in size was detected only in colon cancer cell line SW480 by Northern blot analysis.

gosity is frequent in human cancer, nucleotide sequence of *FGF-20* in normal tissues, human primary gastric cancers, and human primary breast cancers were analyzed by direct sequencing of cDNA-PCR products. Nucleotide substitution or deletion was not detected in normal human tissues (CLONTECH) or surgical specimen of primary gastric cancer or breast cancer examined in this study.

DISCUSSION

In this paper, we have cloned and characterized human *FGF-20*, which encodes a 211-amino-acid polypeptide with the FGF-core domain (Fig. 1). Among the human FGF family, FGF-20 was found to be most homologous to FGF-9, followed by FGF-16 (Fig. 2). Total amino-acid identities among FGF-20, FGF-9, and FGF-16 are as follows: FGF-20 vs FGF-9, 71.6%; FGF-20 vs FGF-16, 66.2%; FGF-9 vs FGF-16, 72.4%. Partial amino-acid identities in the FGF-core domain among FGF-20, FGF-9, and FGF-16 are as follows: FGF-20 vs FGF-9, 76%; FGF-20 vs FGF-16, 71%; FGF-9 vs FGF-16, 78%; however, partial amino-acid identities in the FGF-core domain between FGF-20 and FGFs other than FGF-9 and FGF-16 are less than 50%. Phylogenic analysis indicated that FGF-20, FGF-9, and FGF-16 constitute a subfamily among the human FGF family (Fig. 2).

Hydropathy analysis showed that FGF-20 contains a strong hydrophobic region in the FGF-core domain, but no typical N-terminal signal sequence (Fig. 1C). These structural features are shared by FGF-9 and FGF-16. Despite the absence of a typical N-terminal signal sequence, FGF-9 is efficiently secreted into the culture medium from expressing cells (19). Both an atypical noncleaved signal sequence within the N-terminal 28-

B

Exon or Intron	Size (bp)	Nucleotide sequence
Exon 1	>419	5'-UTR ..... AGCCTCRRCG
Intron 1	5988	gtacgtacta ..... acatatgcag
Exon 2	104	GTATCTTGGA ..... CTATGGATCA
Intron 2	2338	gtacgtatta ..... tctctttcag
Exon 3	>493	GAGAAACTTA ..... 3'-UTR

FIG. 4. Structure of the human *FGF-20* gene. (A) Exons are indicated by boxes. The coding region and the UTRs are indicated by the closed and open boxes, respectively. (B) Nucleotide sequence of the *FGF-20* gene around the exon-intron boundaries. Nucleotide sequence of the *FGF-20* gene in exon is indicated by capital letters, while that in intron is indicated by lowercase letters. Exon sequence of the *FGF-20* gene is determined in this study (Accession No. AB AB044277). Intron sequence of the *FGF-20* gene is derived from the human genome sequence of the chromosome 8p21.3-p22 region (Accession No. AB020858).

amino-acid sequence and a hydrophobic region in the FGF-core domain are important for microsomal translocation and secretion of FGF-9 (34, 35). We propose that this secretion mechanism of FGF-9 might be shared by FGF-16 and FGF-20.

FGF-20 mRNA of 2.4 kb in size was detected only in SW480 cells by Northern blot analysis (Fig. 3). *FGF-16* is predominantly expressed in the heart and brown adipose tissue (22). *FGF-9*, isolated from the conditioned medium of a human glioblastoma cell line, is predominantly expressed in kidney and brain, especially in neurons of cerebral cortex, hippocampus, substantia nigra, motor nuclei of the brainstem, and Purkinje cell layer (19). *FGF-20*, *FGF-9*, and *FGF-16* might be expressed in the mutually exclusive manner.

Comparison between the *FGF-20* cDNA and the human genome sequence AB020858 indicated that the *FGF-20* gene consists of three exons, and the coding region of the *FGF-20* mRNA is dispersed on three exons (Fig. 4).

Human chromosomal localization of other *FGF* genes are as follows: FGF-1, 5q31; FGF-2, 4q25; FGF-3, 11q13; FGF-4, 11q13; FGF-5, 4q21; FGF-6, 12p13; FGF-8, 10q24; FGF-10, 5p12-p13; FGF-11, 17p12-p13; FGF-12, 3q28; FGF-18, 14p11; FGF-19, 11q13.1 (36–45). In this study, the *FGF-20* gene was found to be located on 8p21.3-p22, where loss of heterozygosity is frequent in human cancer.

C at the nucleotide position 329 of the *FGF-20* cDNA was found to be deleted in the corresponding nucleotide position 16021 of the human genome sequence AB020858. This deletion in the coding region of the *FGF-20* mRNA leads loss of the latter half the FGF-core domain due to the frameshift. Nucleotide sequence analysis of the *FGF-20* cDNA by direct-sequencing of cDNA-PCR products did not show deletion of C at the nucleotide position 329 of the *FGF-20* cDNA in any RNA samples examined in this study. One nucleotide deletion at the nucleotide position 16021 of the human genome sequence AB020858 might be a sequencing error.

C at the nucleotide position 277 of the *FGF-20* cDNA was found to be substituted to G at the nucleotide position 16071 of the human genome sequence AB020858, but not in any normal tissue samples (CLONTECH) examined in this study. The C → G substitution at the nucleotide position 277 of *FGF-20* cDNA in the human genome sequence AB020858 might be a single nucleotide polymorphism of the *FGF-20* gene rather than a point mutation of the *FGF-20* gene in SW480 cells.

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